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Abstract

Samples of conventional (n = 11) and organic (n = 81) milk, both raw and heat-treated, were analysed for the presence of synthetic antioxidants (butylated hydroxytoluene, butylated hydroxyanisole, dodecyl gallate, propyl gallate and octyl gallate) to verify whether those labelled as "organic" corresponded to EU Regulations on the use of additives in such products. The analysis detected only the antioxidant BHT and its aldehyde BHT–CHO in all 11 conventional milk and in 18 of 81 organic milk samples. The investigation highlights the importance of strict control of organic dairy production, since synthetic antioxidants added to feedstuff to prevent rancidity can be transferred to milk.

Keywords

BHA; BHT; GC–MS; HPLC; Organic milk; Synthetic antioxidants

1. Introduction

Increasing consumer demand for healthy food has gone apace with greater attention to production methods that respect the environment and animal welfare. Parallel to the rising popularity of foodstuffs produced by organic methods is the annual growth rate of 20–40% in the number of organic farms (Sato, Bartlett, Erskine, & Kaneene, 2005) in Europe from approximately 8000 in 1985 to 142,000 in 2001 (Hermansen, Strudsholm, & Horsted, 2004). Italy ranks highest in the number of organic farms, accounting for 10.3% of total production in 2003 (http://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/442), compared with only 1.4% of the total organic dairy sector in Europe (Thomassen, Van Calker, Smits, Iepema, & De Boer, 2008). Over the last decade, growth rates in the organic dairy market and consumption varied considerably between countries, reaching 14% of the total dairy market in Denmark and over 25% in Switzerland (Sato et al., 2005).

In 1991, specific EU laws were enacted to regulate organic production (Council Reg. EEC 2092/1991). A major issue these regulations address concerns the use of additives in feedstuffs for animals producing organic meat and milk. Except for E306 tocopherol-rich extracts of natural origin (Council Reg. EEC 2092/1991), the list of synthetic substances used as antioxidants, but banned from organic production, includes butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG) and dodecyl gallate (DG). Thanks to their high-performance, low cost and wide availability, these substances are frequently used in the processing of feedstuffs and food (Fki et al., 2005, Guo et al., 2006, Nenadis et al., 2003, Perrin and Meyer, 2002 and Rafecas et al., 1998). Their effects on humans have been extensively studied, though sometimes with controversial results (Bianchi et al., 1997, Botterweck et al., 2000, FAO/WHO Expert Committee on Food Additives, 1986, Iverson, 1995, LeClercq et al., 2000 and Whysner and Williams, 1996).

While BHT is also extensively used in non-edible material, it has been reported only as an air contaminant. Since in soil and water it transforms into substances at low permanence in the environment (http://www.inchem.org/documents/sods/sids/128370.pdf; http://www.jetoc.or.jp/HP_SIDS/pdffiles/128-37-0.pdf), certain quantities of BHT may be found in feed and food as well.

Here, we report the first results of an investigation that analysed organic and conventional milk samples for the presence of synthetic antioxidants (BHA, BHT, PG, OG and DG).

2. Materials and methods

2.1. Materials

The analysis was performed on 92 milk samples: 75 samples (36 cow, 27 sheep and 12 goat milk) came from certified organic farms and one from a conventional farm. Milk samples from the retail market, both organic (six samples) and conventional (10 samples), were also analysed (Table 1). The samples from the organic farms were collected in winter (January–February) and in summer (July–August).

Table 1.

Types of milk analysed.

Sample no.	Organic milk	Sample number	Conventional milk
O1–O2	Pasteurized whole milk	C1	Raw whole milk
O3	Pasteurized partially skimmed	C2–C3–C8–C10–C11	Pasteurized whole milk
O4	UHT whole milk	C4	Pasteurized partially skimmed
O5	Pasteurized whole milk	C5–C6–C7–C9	UHT whole milk
O6	Pasteurized whole milk		
O7–O81	Raw whole milk		

2.2. Reagents

All reagents were HPLC grade Merck (Darmstadt, Germany), and water was obtained by means of a MilliQ apparatus Millipore (Billerica, MA, USA). The standards of BHT, BHA, PG, DG and OG were purchased from Sigma (St. Louis, MO, USA). BHT aldehyde (BHT–HO) was purchased from ABCR GmbH and Co., (Karlsruhe, Germany). Aldehyde of BHT was also analysed because it is a metabolite of BHT (Fries & Püttmann, 2002).

2.3. Analytical methods

Extraction was done on lyophilised samples using methanol saturated with hexane as follows: 50 ml of lyophilised sample was redissolved in 50 ml of ultrapure water; samples were extracted twice with 50 ml of hexane saturated with methanol at 60 C for 30 min; the hexane was then collected and extracted five times with 15 ml of methanol, each time mixing for 1 min at 60 C; the methanol was then separated and collected using a separation funnel. After evaporation, the extract was redissolved in 2.5 ml of methanol and injected into the column. The analytical conditions were those described by Pinho, Ferreira, Oliveira, and Ferreira (2000) with minor modifications (flow rate 1.5 ml/min instead of 2.0 ml/min). Methods blanks were analysed every day of the analysis.

The gas chromatographic (GC) run was set up as follows: the GC injector temperature was 250 C; the GC oven temperature was initially held at 70 C for 1 min, then increased by 50–200 C/min (final temperature held for 1 min) and then by 20–320 C/min (final temperature held for 0.50 min). The transfer line was heated to 280 C and the mass selective detector was operated with an electron impact source.

Both the batch and the standard solutions for analyte identification and characterisation and for calibration were prepared in methanol as follows: 1 μl of the different solutions was injected into the GC injector operating in splitless mode.

The analyte solutions were first injected in full scan mode to evaluate retention times and mass spectra characteristics which were identified by comparing them with mass spectra databases (NIST 1998). A selected ion monitoring (SIM) method was created to improve instrument sensitivity, with recording of the mass spectra acquiring only the most intense ions in the mass spectra obtained in full scan mode (205 and 220 m/z for BHT and 191, 219 and 234 m/z for BHT–CHO).

2.4. Instruments

Separation and quantification were performed on an HPLC LaChrom Merck–Hitachi (Darmstadt, Germany) HPLC apparatus equipped with a L-7000 pump, a UV detector set to 280 nm, a PuroShper RP C-18 column. The analytes were confirmed by means of a 6890N Network Gas chromatographic System coupled with a 5973 inert Mass Selective Detector Agilent (Santa Clara, CA, USA) operating in fast GC mode. The GC column was a DB1-ms (5 m × 0.1 mm × 0.1 μm).

3. Results and discussion

Milk is a notoriously complex matrix to analyse. In order to obtain a clearer chromatogram, we tried different percentages in mobile phases (data not shown) and ultimately decided to use a slower speed rate. The wavelength was decided after the analysis of the absorbance spectrum of each standard. All standard curves showed good linearity ($R^2 > 0.99$). The detection limit was 1 ppb. The recoveries at a concentration of 0.25 ppm were 61.5 ± 1.2%, 76.3 ± 5.1% and 77.7 ± 1.4% for BHT, BHA and BHT–CHO, respectively, and 84.3 ± 7.5%, 52.1 ± 6.7% and 54.7 ± 7.6% at a concentration of 5 ppm. The recovery of gallates was <50% at both concentrations.

Since none of the samples resulted positive for gallates or BHA, only BHT and BHT–CHO were entered into the analysis. Nonetheless, due to the low recovery rates for gallates, their possible presence cannot be completely excluded.

Table 2, Table 3 and Table 4 list the results of the positive samples.

Table 2.

BHT and BHT–CHO in conventional milk analysed by HPLC.

Sample no.	BHT (μg/100 ml of milk)	BHT–CHO (μg/100 ml of milk)
C1	130.4 ± 15.0	30.4 ± 3.4
C2	n.d.	1.6 ± 0.1
C3	n.d.	2.0 ± 0.2
C4	63.8 ± 15.6	3.6 ± 0.4
C5	n.d.	3.2 ± 0.2
C6	11.6 ± 4.8	1.6 ± 0.6
C7	12.4 ± 3.8	7.8 ± 0.6
C8	127.8 ± 6.4	n.d.
C9	33.6 ± 2.8	1.6 ± 0.4
C10	7.6 ± 2.4	14.2 ± 1.2
C11	42.6 ± 9.8	29.0 ± 2.4

Plus–minus values are the means ± SD.

n.d.: not detected.

Table 3.

BHT and BHT–CHO in milk from organic farms.

Sample no.	Animal species	Sampling period	BHT (µg/100 ml of milk)	BHT–CHO (µg/100 ml of milk)
O11	Bovine	Winter	8.6 ± 1.6	n.d.
O19	Bovine	Winter	13.2 ± 0.3	2.9 ± 0.2
O25	Bovine	Winter	n.d.	3.1 ± 0.1
O33	Bovine	Winter	11.9 ± 0.3	n.d.
O41	Goat	Winter	27.6 ± 4.2	3.0 ± 0.2
O42	Goat	Winter	28.0 ± 0.5	1.4 ± 0.1
O43	Goat	Winter	29.0 ± 0.9	2.7 ± 0.1
O44	Goat	Winter	28.4 ± 2.9	4.5 ± 0.2
O45	Sheep	Winter	21.9 ± 0.1	2.1 ± 0.1
O52	Bovine	Winter	23.0 ± 1.0	24.0 ± 1.0
O69	Goat	Summer	1.1 ± 0.3	n.d.
O70	Goat	Summer	1.0 ± 0.1	n.d.
O71	Goat	Summer	0.5 ± 0.1	n.d.
O72	Goat	Summer	1.7 ± 0.4	n.d.
O73	Sheep	Summer	0.9 ± 0.1	n.d.

Plus–minus values are the means ± SD.

n.d.: not detected

Table 4.

Organic milk from the retail trade.

Sample no.	BHT (µg/100 ml of milk)	BHT–CHO (µg/100 ml of milk)
O1	n.d	n.d.
O2	n.d.	n.d.
O3	n.d.	n.d.
O4	141.2 ± 26.2	11.8 ± 0.4
O5	40.2 ± 2.8	n.d.
O6	n.d.	4.4 ± 0.2

Plus–minus values are the means ± SD.

n.d.: not detected.

The highest BHT content was found in the milk samples from the conventional farm where cows received feedstuff containing BHT (sample C1) (Table 2). This result was confirmed by GC. We chose fast GC because it is an innovative GC operating mode that uses a very short GC column and very rapid temperature ramps, thus permitting very fast chromatographic runs. The two different aliquots of the milk samples from the same farm where the animals received feed containing BHT (samples C12 and C13) were re-extracted in triplicate for the GC–MS analysis, and injected in triplicate. The mean concentration was 76.7 ± 2.15 µg/100 ml of milk and 141.1 ± 3.73 µg/100 ml of milk for BHT and 39.5 ± 3.32 µg/100 ml of milk and 45.9 ± 1.07 µg/100 ml of milk for BHT–CHO, respectively. The regression equations were $y = 20.1x - 704$ for BHT and $y = 14.6x - 228$ for BHT–CHO, with a correlation coefficient of 0.999 and 0.998, respectively. The retention times (RTs) for the two analytes were 2.00 and 2.50 min for BHT and BHT–CHO, respectively (data not shown). Identification and confirmation of the two analytes were highly accurate (97% for BHT and 94% for BHT–CHO) in comparison to the obtained mass spectra with the database mass spectra (Fig. 1 and Fig. 2).

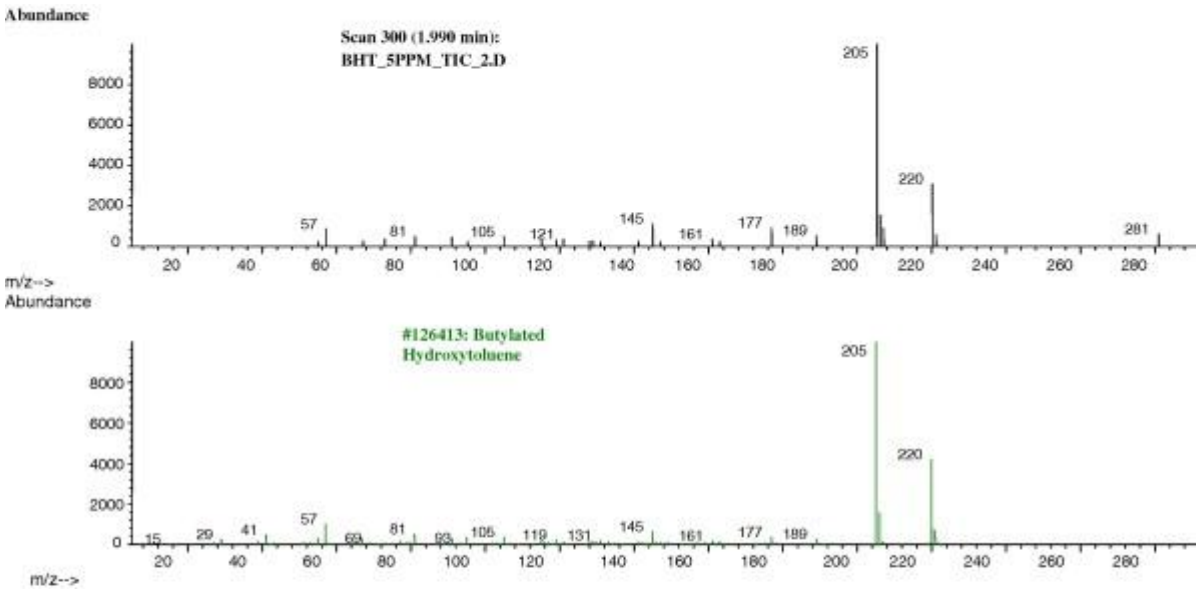

Fig. 1.

Mass spectra of BHT in sample C12 (above) and mass spectra of BHT from NIST 1998 database (below).

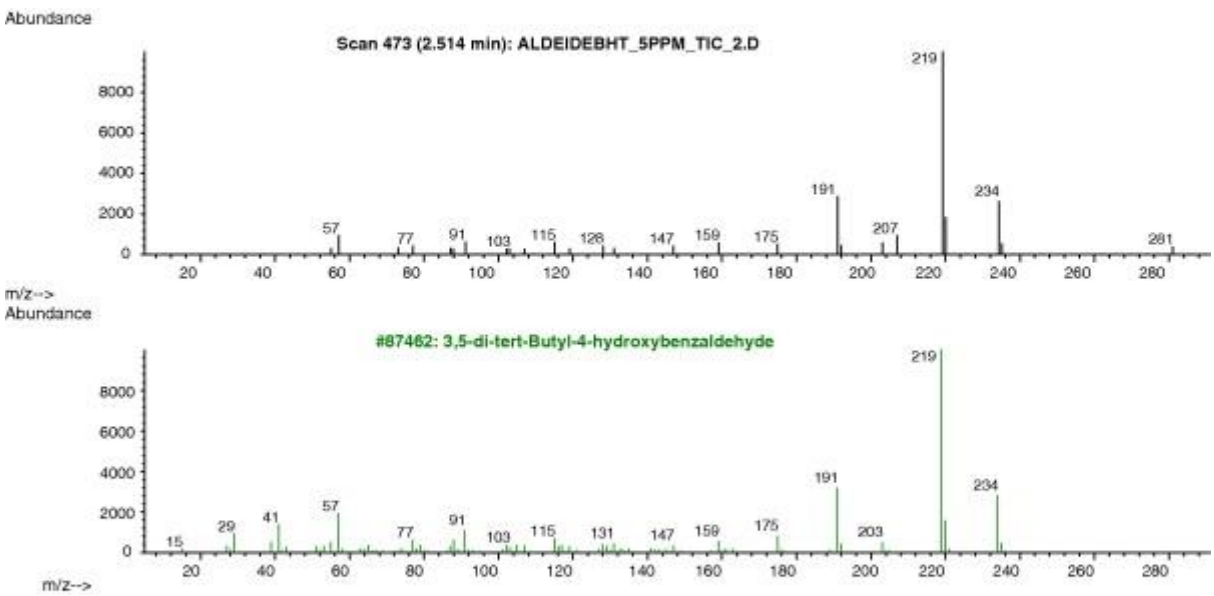

Fig. 2.

Mass spectra of BHT–CHO in sample C13 (above) and mass spectra of BHT from NIST 1998 database (below).

Table 3 and Table 4 illustrate the results of analysis of the organic milk samples. BHT and/or BHT–CHO were detected in 15 of 75 samples. No antioxidants were found in the bovine milk sampled in the summer, and only traces were detected in the goat and sheep milk samples (Table 3). Integration with non-controlled feedstuff in winter due to insufficient pasture may be the reason for these results (Nardone et al., 2004 and Nauta et al., 2006). All the samples came from farms in northern Italy where organic farmers normally use concentrates in order to increase milk production (Häring, 2003). In those formulations, antioxidants, specifically BHT, are frequently used for oil stabilization (Guo et al., 2006). Concentrates cause no end of trouble for farmers. Thomassen et al. (2008) reported that, because of limited choice, farmers can hardly influence the composition of the concentrates they buy. Our analysis of feeds given in winter on three farms confirmed the presence of BHT (1.7–12.5 μg/100 g of feed); BHT–CHO was never detected. The reason for the lower levels of antioxidants in the summer samples of milk could be summer grazing. Since the feed intake of grass versus concentrate is higher, a dilution effect on the amount of BHT ingested by the animals on those farms is not unlikely. Moreover, summer grazing is a common procedure in organic farming (Lund & Algers, 2003).

Of the six organic samples from the retail market, three contained BHT, BHT–CHO or both (Table 4). Unexpectedly, sample O4 presented even higher levels of antioxidants than the maximum amount found in the conventional milk samples.

The results emphasise the importance of more meticulous controls in the certification of organic production, more controls across all steps of the food and feed chain, and more transparency (Nardone et al., 2004 and Vaarst et al., 2005).

Toxicology studies and research into the metabolic and the excretion pathways of antioxidants focus on the urinary system, fat accumulation and liver conjugation, as emunctory apparatus and accumulation sites (http://www.inchem.org/documents/sods/sids/128370.pdf; http://www.jetoc.or.jp/HP_SIDS/pdffiles/128-37-0.pdf). The finding of small amounts of antioxidants in milk suggests that the mammary gland could be included as an emunctory organ.

As concerns human consumption, the acceptable daily intake (ADI) for man established by the Scientific Committee of Food in 1987, revised in 1989, for BHT is 0.05 mg/kg body weight, which is six times lower than 0.3 mg/kg allocated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Soubra, Sarkis, Hilan, & Verger, 2007). In Italy, the daily consumption of milk is 213 g/die/person (http://www.cpo.it/documenti/alimentazionesito.PDF). Based on the results of our analysis, the estimated amount of BHT ingested would be far lower than either of these ADI values. Furthermore, taking 60 kg as adult mean weight, milk would make up only 1% of the ADI. We feel, therefore, that our results do not necessarily raise concern for human consumption. However, BHT and other antioxidants may be dangerous when, besides dairy products, the consumption of other foods like biscuits, candies and chewing-gum is tallied into the account (Soubra et al., 2007).

4. Conclusion

Consumer demand for healthier food from productions that respect the environment and animal welfare has boosted the growth of organic farming over the last 20 years. To date, the feed and food chain is controlled only through certification. Despite European regulations banning the use of synthetic antioxidants in organic farming, they are often added to animal feed as an economical way to prevent oxidation.

In the 92 samples of conventional and organic milk we analysed, BHT and BHT–CHO were found in all the conventional milk samples, in 18 of the 81 organic milk samples, as well as in the animal feed from the organic productions, probably because production during winter is too low unless the feedstuff is integrated with concentrates.

The toxicology analysis showed that even small amounts of these substances can be transferred to milk by the mammary gland. Nonetheless, even the highest amount found in the conventional milk did not exceed currently established ADI values. Even so, our results point to the need for stricter feedstuff control, greater transparency and control of the feed and food chain, and more stringent certification requirements.

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